Discrimination of Hoechst Side Population (SP) Cells in Mouse Bone Marrow using a NPE Quanta Analyzer Equipped with a Mercury Arc Lamp or a Near-UV Laser Diode

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Abstract

Discrimination of stem cells (side population, or "SP" cells) by flow cytometric analysis of Hoechst 33342 efflux is a valuable methodology for identifying these cells in progenitor enriched bone marrow. Unfortunately, it requires a UV laser source, usually necessitating an expensive and maintenance-intensive argon- or krypton-ion ion laser on a large flow cytometer. In the present study, we have used a NPE Quanta Analyzer, (a low-cost flow cytometer equipped with a UV-emitting HBO arc lamp and a Coulter transducer flow cell for measurement of cell volume) for the detection of bone marrow SP cells. In addition to making SP analysis more accessible via a lower cost instrument platform, the ability to analyze the electronic cell volume of SP cells may provide a valuable new phenotypic marker for stem cell analysis.

The NPE analyzer in this study was equipped either with a 120 W HBO arc lamp and one of several UV excitation filters (including Schott and Hoya colored glass and shortpass dichroics and narrow bandpass UV filters), or with a near-UV laser diode (NUVLD) emitting at 374 nm, 8 mW (Power Technology, Alexander, AR. Two high-sensitivity Hamamatsu PMTs using broad bandpass or longpass filters were used for detection of the Hoechst blue and red fluorescent signals. SP cells were detectable on the NPE Analyzer using the HBO arc source, but the resolution was somewhat lower and less reproducible than that seen with laser sources on traditional flow cytometers. NUVLD excitation produced a side population with somewhat better resolution and separation from non-SP cells.

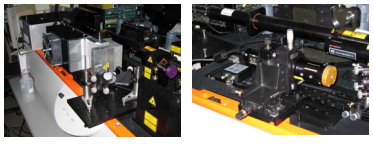
Low UV power at the flow cell (estimated to be less than 2 mW) appears to be one reason for less-than-optimal resolution with the HBO arc source; while this is more than adequate for traditional DAPI DNA content analysis, the Hoechst red emission in particular appeared to require higher levels of UV excitation than that provided by the Hg source in its present configuration. Red and near-IR emission from the HBO lamp also appeared to saturate the Hoechst red PMT and reduce SP resolution. Efforts are now being made to increase the UV power level at the flow cell, reduce red light contamination by the Hg source, and increase detector sensitivity to improve SP resolution on this instrument with both HBO and laser excitation sources.

Materials and Methods

Mice and cells. BALB/c female mice four to twelve weeks of age (Jackson Laboratory, Bar Harbor, ME) were maintained in the NIH-NICHD single pathogen free barrier animal colony, and were euthanized immediately prior to bone marrow aspiration according to NIH guidelines. Bone marrow was extracted by fine needle aspiration and washed twice with HBSS containing 2% FBS and 10 mM HEPES. Some bone marrow samples were initially incubated with anti-Fcγ2/Fcγ3 antibody for ten minutes at 4°C, followed by incubation with PE-conjugated antibodies against the lineage markers B220, Ter-119, CD3, Gr-1 and Mac-1 (BD Pharmingen, San Diego, CA) for twenty minutes at 4°C. Cells were washed and subsequently incubated with anti-PE antibody conjugated paramagnetic beads (Miltenyi Biotec, Auburn, CA) for twenty minutes. Lineage-positive cells were then removed using an AutoMACS cell separation unit (Miltenyi Biotec), using the normal depletion program. The lineage-positive (SP-depleted) and lineage-negative (SP-enriched) populations were washed in the above buffer and counted. A549 lung carcinoma cells were obtained from the American Red Cross (Rockville, MD) and passaged in Dulbecco's MEM containing 10% FBS. These cells were removed from their growth substrate with trypsin/EDTA, washed with cold HBSS/FBS/HEPES and counted prior to Hoechst 33342 labeling.

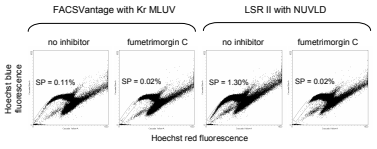
Hoechst 33342 labeling for SP discrimination. The above cell fractions, unseparated bone marrow and A549 cells were then labeled with Hoechst 33342 using the method previously described by Goodell et al. (1,3). Briefly, cells were resuspended at 107 cells per ml in HBSS with 2% FBS and 10 mM HEPES and prewarmed to 37°C. For some samples, bone marrow cells were preincubated with the ABCG2 inhibitor fumitremorgin C at 10 μM for fifteen minutes. Hoechst 33342 was then added at a final concentration of 5 μg/ml and the cells incubated for 90 minutes at 37°C with periodic mixing. Cells were washed with cold HBSS/FBS/HEPES and stored on ice until analysis (within four hours).

Successful Hoechst SP labeling was confirmed for all samples prior to NPE Quanta analysis using either a BD FACSVantage equipped with a krypton-ion laser emitting a multiline UV beam at 100 mW, or a BD Biosciences LSR II equipped with either a near-UV laser diode (NUVLD) emitting at 374 nm, 8 mW, or a Nd-YAG frequency-tripled quasi-CW laser emitting at 355 nm, 22 mW. A standard filter configuration using a 450/50 nm bandpass for Hoechst blue, 650 LP for Hoechst red, and a 580-610 SP dichroic to split the signals was used for all analyses.



UV laser sources for Hoechst SP analysis on the BD LSR II. *Left*, Lightwave Nd-YAG mode-locked 355 nm laser. *Right*, Coherent near-UV 374 nm laser diode.

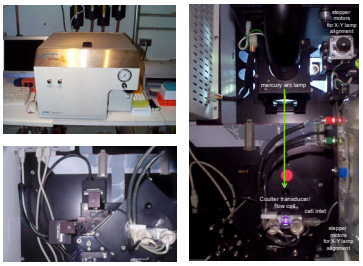
Hoechst SP analysis on conventional flow cytometers



Hoechst SP analysis on conventional flow cytometers. *Left two histograms* analysis on FACSVantage with Kr MLUV 100 mW excitation; *right two histograms*, Analysis on BD LSR II with NUVLD 374 nm 8 mW.

Hoechst SP analysis with Hg HBO excitation

Mouse bone marrow labeled for Hoechst SP was initially analyzed on the NPE Quanta Analyzer equipped with a mercury arc lamp emitting at 80 mW, with several UV filters interposed between the lamp and the flow cell (including UV bandpass filters, Schott and Hoya UV glass filters). A dual longpass dichroic reflected the UV to the flow cell, and transmitted both blue and red signals to the PMTs. The instrument was equipped with two Hamamatsu red-sensitive PMTs; a 450/50 nm bandpass filter was used for Hoechst blue detection and a 650 LP for Hoechst red, with a 610 SP dichroic to split the signals. Instrument alignment sensitivity assessment was carried out using both DAPI-labeled trout red blood cells (TRBCs) and Molecular Probes InSpeck Blue sensitivity microspheres, with alignment confirmed through both through the blue and red detectors.

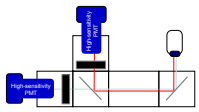


Above left, NPE Quanta Analyzer

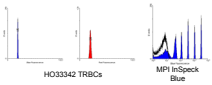
Right, Optical bench configuration of NPE unit,showing mercury arc lamp, automatic lamp and flow cell alignment system, and Coulter flow cell. A variety of UV exciter optics were tested including bandpass filter and Schott and Hoya glass UV filters with varying long red emission properties.

Lower left, PMT configuration of NPE Quanta Analyzer. Left-most PMT was used for Hoechst blue detection (450/50 nm bandpass), upper PMT for Hoechst red detection (650 LP filter). Signals were split with a 610 SP dichroic.

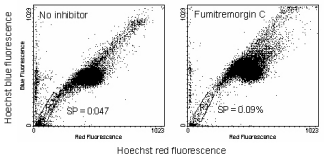
Upper left, conventional optical bench configuration for DNA content analysis.



Lower left, modified optical bench configuration for Hoechst SP analysis, with two detector positions equipped with Hamamatsu red-sensitives PMTs. The conventional filter configuration for Hoechst SP analysis could then be used.



Lower left, alignment standards for Hoechst SP analysis on the NPE Quanta Analyzer. Left histogram, Hoechst 33342 labeled trout red blood cells (TRBCs) analyzed through the Hoechst blue channel (450/50 nm); *middle histogram*, Hoechst 33342 labeled TRBCs analyze through the Hoechst red channel (650 LP); *right histogram*, Molecular Probes InSpeck Blue microspheres analyzed through the Hoechst blue channel, as a measure of instrument sensitivity.



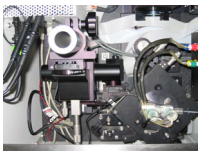
Hoechst SP analysis on the NPE Quanta Analyzer with Hg HBO lamp. *Left histogram*, no inhibitor. *Right histogram*, with fumetrimorgin C at 10 uM.

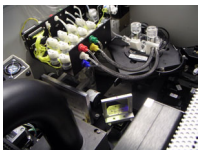
Problems

- Hoechst SP detectable but with highly variable resolution.
- Mercury lamp excitation in its present configuration was probably suboptimal – it is estimated that less than 1 mW (< 1 mJ) UV light actually interrogates the flow cell
- Near-IR light from the Hg lamp interfered with Hoechst red resolution and increased background – modification of UV exciter to reduce this interference (i.e. use of Hoya glass filters) only partially resolves this problem

Hoechst SP analysis on NPE with NUVLD

In an attempt to improve SP resolution on the NPE Quanta Analyzer, a near-UV laser diode (NUVLD) was installed as an excitation source in place of the Hg HBO source. This laser emitted at 374 nm, 8 mW, a wavelength and power previously found to give excellent Hoechst SP resolution on conventional instruments.

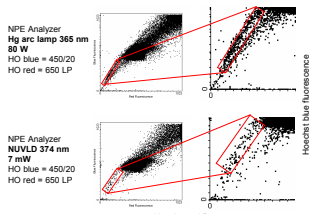




Left, Power Technology NUVLD.

Upper right, temporary installation on NPE Quanta Analyzer with four-axis Alignment.

Lower right, permanent installation on NPE Quanta Analyzer. Large laser Dichroic permited simultaneous use Of Hg HBO excitation in the green.



Hoechst SP analysis using either Hg HBO or NUVLD sources. Mouse bone marrow labeled with Hoechst 33342. *Top cytograms*, Hg HBO source; bottom cytograms, NUVLD. NUVLD excitation gave somewhat better discrimination of Hoechst SP cells from non-stem cell sub-G1 events.

NUVLD excitation produces somewhat better resolution of Hoechst SP, but still somewhat short of that found on conventional cuvette flow cytometers

Work is continuing in optimizing both near-UV laser and Hg HBO sources as a cost-effctive technology for Hoecht SP analysis.